Discovery of *Juniperus sabina* var. *balkanensis* R. P. Adams and A. N. Tashev in Albania and relictual polymorphisms found in nrDNA

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ABSTRACT

Additional analyses of trnS-trnG and nrDNA from specimens from Albania revealed the presence of *J. sabina* var. *balkanensis*. Careful chromatogram analysis of eight (8) polymorphic sites in nrDNA revealed that 6 of the 8 plants were polymorphic (RSRMYRK or RSRMARK) and apparently hybrids between type 1 GGACCCAG and type 2 (ACGATAGT) plants. Two plants were homozygous for the 8 sites and they both had the type 1 pattern (GGACCCAG). The type 2 pattern (ACGATAGT) was not found, but due to the presence of hybrids, it probably grows in the area. These two nrDNA types appear to have arisen via hybridization with a *J. thurifera* ancestor. The two types often are found in both v. *sabina* and v. *balkanensis* populations. Published on-line www.phytologia.org *Phytologia 100(2): 187-194 (Sep 22, 2018).* ISSN 030319430.
KEY WORDS: *Juniperus sabina var. balkanensis, J. sabina*, distribution, nrDNA, trnS-trnG, chloroplast capture, ancient nrDNA heterozygotes.

Recently, Adams et al. (2018) reported on new populations of *J. sabina* var. *balkanensis* from Macedonia, Bosnia-Herzegovina, Croatia and central Italy (Fig. 1). As a part of this continuing study (Adams et al. 2016, 2017, 2018; Adams 2014), we now report on the confirmation of *J. sabina* var. *balkanensis* from Albania.

MATERIAL AND METHODS

Specimens used in this and previous studies: (species, popn. id., location, collection numbers): See Adams et al. (2018) for previously analyzed specimen locations.
Albania: North-Eastern Albanian Alps, above the road from Ceren to Radomira village, 41° 49' 43.86" N; 20° 27' 43.05" E. ca. 1150 m and above the village of Ceren, on path to Sorokol, 41° 49' 37.01" N; 20° 28' 28.13" E. ca. 1430 m**.** Coll. *Lulëzim Shuka s.n.* 22 June 2018. Lab Acc. *Adams 15506-15513* (8 samples). Voucher specimens for all collections are deposited at Baylor University Herbarium (BAYLU).

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS primers utilized. The primers for trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010). The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. 2.31 (Technelysium Pty Ltd.).

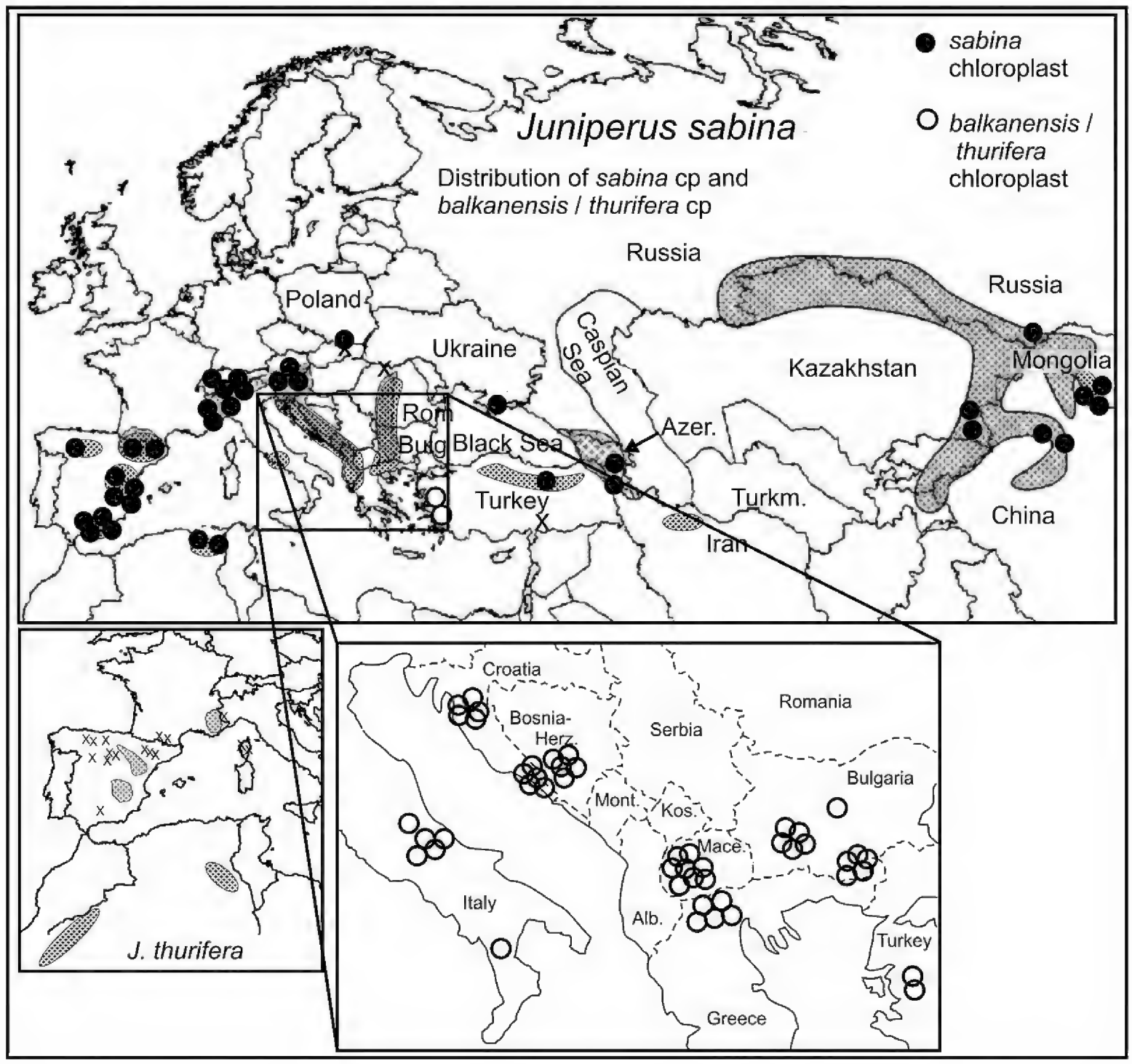

Figure 1. Distribution of *J. sabina* var. *balkanensis* and typical *J. sabina* chloroplast. The present day distributions of *J. thurifera* and var. *africana* (in north Africa) are shown in the insert on the lower left. (modified from Adams et al, 2018).

RESULTS

All 8 plants sampled in Albania were *J. s.* var. *balkanensis* based on cp DNA (Table 1). The revised distribution of v. *balkanensis* is shown in Figure 2. All known locations of v. *balkanensis* are in a relatively small geographical area.

Before discussing the nrDNA patterns, it should be noted that there are recent papers analyzing the inheritance of nrDNA in the Cupressaceae. Adams and Matsumoto (2016) analyzed 3 variable sites of nrDNA from synthetic crosses between *Cryptomeria japonica* cv. Haara and cv. Kumotooshi (= cv. Haara x Kumotooshi, ie., a backcross). They found that 3 of the 7 progeny had nrDNA very similar to that of the Haara x Kumo parent. In contrast, 4 of the 7 progeny had nrDNA exactly like the Haara parent. This appears to suggest that nrDNA polymorphisms can revert to that of a recurrent parent in the case of backcrossing.

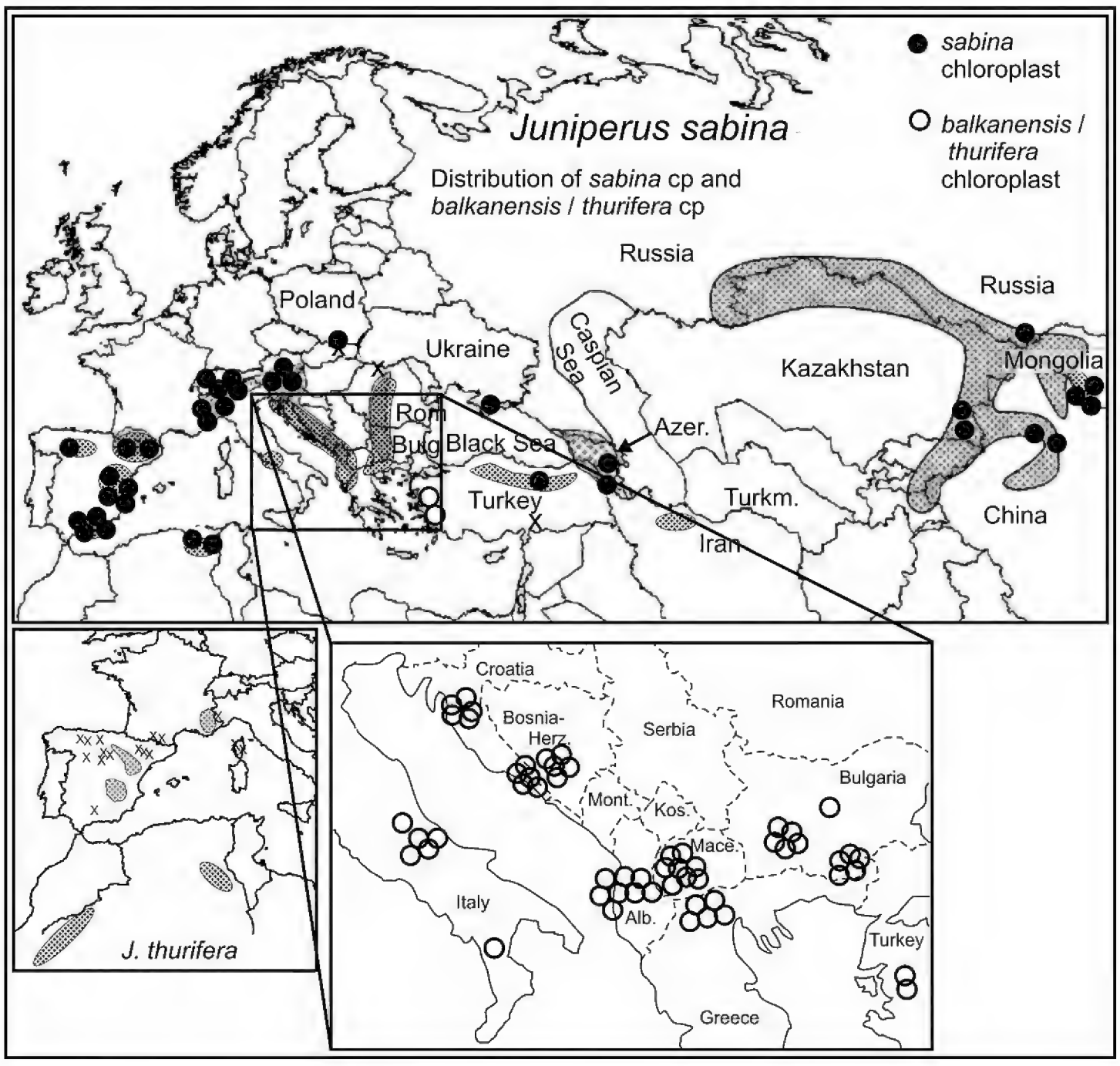

Figure 2. Revised distribution of *J. sabina var. sabina* (dark circles) and *J. s.* var. *balkanensis* (open circles) based on cp DNA (present study and from Adams et al. 2018)

Adams, Miller and Low (2016) examined 8 variable nrDNA sites in the parents (*Hesperocyparis arizonica*, *H. macrocarpa*), and their 18 artificial hybrid progeny. Each of the 18 hybrids were heterozygous for all 8 nrDNA sites. This study is very relevant to the present study, because *Hesperocyparis* (= *Cupressus* in the western hemisphere) is very closely related to *Juniperus* (Little et al. 2004, Terry, et al. 2012, Terry and Adams, 2015, Terry et al. 2016) and because there are no verified artificial hybrids of *Juniperus* available to the authors for the examination of the inheritance of nrDNA in *Juniperus*, the Adams, Miller and Low (2016) research on *Hesperocyparis* stands as a proxy for the inheritance of nrDNA in *Juniperus* and thus, their study in *Hesperocyparis* is surely applicable to *Juniperus*. So, we can confidently assume that nrDNA is inherited by complementation as found in *Hesperocyparis*, *Cryptomeria* and all other conifers. It should be noted that Adams, Miller and Low (2016) sequenced cp markers and confirmed that the cp genome is inherited via pollen (paternally inherited) in *Hesperocyparis* (and presumably *Juniperus*).

Aligning *J. sabina* and *J. thurifera* nrDNA sequences revealed that the taxa differ by SNPs at 22 sites, all in ITS1 or ITS2. Previously (Adams et al. 2018), a close examination of the nrDNA sequencing chromatograms revealed that generally, only 8 of the 22 sites contained heterozygous peaks. The 8 sites were (with position): 352(R), 391(S), 432(R), 606(M), 745(Y), 999(M), 1046(R), 1047(K). Two nrDNA types were found considering these 8 sites. Both of these types, type 1 (GGACCCAG) and type 2 (ACGATAGT), have been found in var. *balkanensis* and in var. *sabina* populations. Adams et al. (2018) reported the majority of the plants examined (42/62) were heterozygous for 1 to 8 sites, (Table 1). Note that 33/ 42 heterozygous individuals were heterozygous for all 8 sites. They reported the frequency of heterozygous sites was: 1,1,1,3,2,1,0,33 (for plants containing from 1 to 8 polymorphic sites, respectively).

In the Albania population, 2 plants (15508, 15511) were homozygous (for the 8 loci) and had the type 1 pattern: GGACCCAG. The other 6 plants were mostly heterozygous (Table 2). Of the 6 heterozygous plants, 15509 and 15510 were RSRYARK (7/8 heterozygous positions), and 15506, 15507, 15509, 15510, 15512, 15513 were RSRMYMRK (8/8 heterozygous positions) (Fig. 3). The type 2 ITS pattern (ACGATAGT, Adams et al. 2018) was not found among these 8 samples, but it surely must be present nearby, as the hybrids (between the 2 ITS types) were common.

Throughout the range of *J. sabina*, heterozygous plants are somewhat randomly distributed (Fig. 3). It is interesting that 4/4 Spain and 2/3 Switzerland plants were homozygous for the 8 sites. Only 2/7 plants from the far East were homozygous. Plants of var. *balkanensis* seem to be a bit more heterozygous (36/48 were heterozygous) than var. *sabina* (Fig. 3, Table 2).

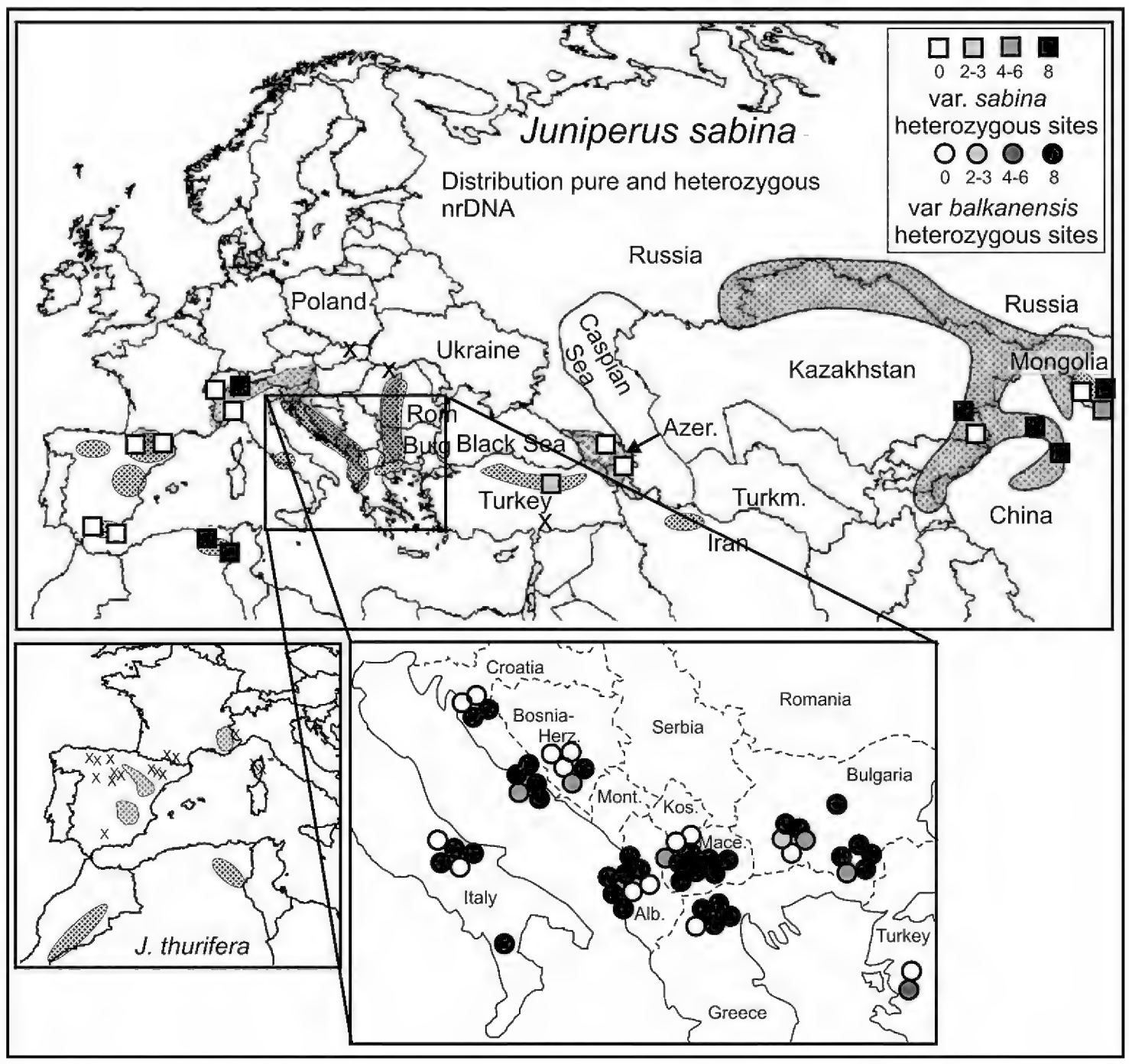

Figure 3. Distribution of homozygosity and heterozygosity among the 8 nrDNA polymorphic sites.

A more detailed examination of nrDNA type 1 (16/20) and type 2 (4/20) homozygous plants (Table 2) shows that both type 1 and type 2 plants were present in the Switzerland samples. One type 2 plant was found in Rila Mtn., Bulgaria and 2 were from Azerbaijan. All 16 type 1 nrDNAs share 4 bp sites with *J. thurifera*, and the four type 2 plants share 3 bp sites with *J. thurifera* (Table 2). It is strange that type 1 nrDNA contains 4 bp, typical of *J. thurifera* (in the 8 sites), and type 2 contain 3 different bp typical of *J. thurifera* (below and Table 2), but these are mutually exclusive in types 1 and 2.

most common sabina pattern (type 1)	G **G** A **C** *C* **C** A **G**	4 sites in common with *J. thurifera*
7083, thurifera, France, Morocco[2]	**A G G C T C G G**	
2nd common sabina pattern (type 2)	**A** C **G** A **T** A **G** T	4 sites in common with *J. thurifera*

Crossing type 1 (GGACCCAG) x type 2 (ACGATAGT) gives RSRMYMRK (see below), which is the same as the putative hybrids (below and Table 1) of RSRMYMRK. Our data indicates that crossing between types 1 and 2 nrDNA types seem common, 46/70 plants were RSRMYMRK. These two nrDNA types may have arisen via hybridization with a *J. thurifera* ancestor and subsequent backcrossing to *J. sabina.*

most common sabina pattern (type 1)	G G A C C C A G
2nd common sabina pattern (type 2)	A C G A T A G T
cross between *J. sabina* type1 x type2	R S R M Y M R K
Putative 'hybrid' pattern (table 1)	R S R M Y M R K

At our present level of understanding, the distributions of *J. s.* var. *balkanensis* and *J. thurifera* do not appear to overlap, negating modern hybridization. However, there were large changes in plant distributions in the Pleistocene and earlier, it seem probable that *J. thurifera*-like ancestors were sympatric with *J. sabina,* and presenting opportunities for chloroplast capture from *J. thurifera.*

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Table 1. Survey of *J. sabina* and classification based on ITS and trnS-trnG sequences. Putative hybrids and backcrosses [*J. sabina* x ancestor of *J. thurifera*] based on ITS polymorphisms are in **bold**.

coll. #, location	trnS-trnG classification (ie. cp genome)	ITS classif.	polymorphic sites[1] 1 2 3 4 5 6 7 8	ITS #poly/ 8 sites
14861 Spil Dağı, Turk., Boratynski	**v. *balkanensis***	***sabina***	**R G R C Y C R G**	**4**
14934 Spil Dagi, Turkey, Mataraci	v. *balkanensis*	*sabina*	G G A C C C A G	0
13725 eastern Rhodopes, Bulgaria	**v. *balkanensis***	***sabina***	**G G A M Y M R K**	**5**
13726 eastern Rhodopes, Bulgaria	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
13727 eastern Rhodopes, Bulgaria	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
13728 eastern Rhodopes, Bulgaria	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
13729 eastern Rhodopes, Bulgaria	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
14721 Sokolna reserve, Bulgaria	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
14722 Rila Mtn., Bulgaria	**v. *balkanensis***	***sabina***	**G G A C Y C A G**	**1**
14723 Rila Mtn., Bulgaria	v. *balkanensis*	*sabina*	A C G A T A G T	0
14724 Rila Mtn., Bulgaria	**v. *balkanensis***	***sabina***	**R G A C Y M A G**	**3**
14725 Rila Mtn., Bulgaria	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
14726 Rila Mtn., Bulgaria	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
14727 Tsena Mtn., Greece	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
14728 Tsena Mtn., Greece	v. *balkanensis*	*sabina*	G G A C C C A G	0
14729 Tsena Mtn., Greece	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
14730 Tsena Mtn., Greece	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
14731 Tsena Mtn., Greece	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15311 Mavrovo, Macedonia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15312 Mavrovo, Macedonia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15313 Mavrovo, Macedonia	v. *balkanensis*	*sabina*	G G A C C C A G	0
15314 Mavrovo, Macedonia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15315 Mavrovo, Macedonia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15316 Gaichnik, Macedonia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15317 Gaichnik, Macedonia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15318 Gaichnik, Macedonia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15319 Gaichnik, Macedonia	v. *balkanensis*	*sabina*	G G A C C C A G	0
15320 Gaichnik, Macedonia	**v. *balkanensis***	***sabina***	**R C R A T M R K**	**5**
15277 Mt. Cabulja, Bosnia-Herze.	**v. *balkanensis***	***sabina***	**R S R A Y M R K**	**6**
15278 Mt. Cvsnica, Bosnia-Herze.	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**

15279 Mt. Cabulja, Bosnia-Herze.	v. *balkanensis*	*sabina*	G G A C C C A G	0
15280 Mt. Cabulja, Bosnia-Herze.	v. *balkanensis*	*sabina*	G G A C C C A G	0
15281 Mt. Cabulja, Bosnia-Herze.	v. *balkanensis*	*sabina*	G G A C C C A G	0
15282 Mt. Biokovo, Croatia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15283 Mt. Biokovo, Croatia	**v. *balkanensis***	***sabina***	**G G A C Y M R K**	**4**
15284 Mt. Biokovo, Croatia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15285 Mt. Biokovo, Croatia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15286 Mt. Biokovo, Croatia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15343 Velebit, Croatia	v. *balkanensis*	*sabina*	G G A C C C A G	0
15344 Velebit, Croatia	v. *balkanensis*	*sabina*	G G A C C C A G	0
15345 Velebit, Croatia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15346 Velebit, Croatia	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15365 Calabria area, southern Italy	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15413 Abruzzo area, central Italy	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15414 Abruzzo area, central Italy	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15415 Abruzzo area, central Italy	**v. *balkanensis***	***sabina***	G G A C C C A G	0
15416 Abruzzo area, central Italy	**v. *balkanensis***	***sabina***	G G A C C C A G	0
15414 Abruzzo area, central Italy	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15506 Ceren, Albania	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15507 Ceren, Albania	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15509 Ceren, Albania	**v. *balkanensis***	***sabina***	**R S R A Y M R K**	**7**
15510 Ceren, Albania	**v. *balkanensis***	***sabina***	**R S R A Y M R K**	**7**
15512 Ceren, Albania	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15513 Ceren, Albania	**v. *balkanensis***	***sabina***	**R S R M Y M R K**	**8**
15508 Ceren, Albania	**v. *balkanensis***	***sabina***	G G A C C C A G	0
15511 Ceren, Albania	**v. *balkanensis***	***sabina***	G G A C C C A G	0
7612 Switzerland	v. *sabina*	*sabina*	G C A C C C A G	0
7614 Switzerland	v. *sabina*	*sabina*	A C G A T A G T	0
14938 northeast Turkey Kandemir	**v. *sabina***	***sabina***	**R G R C T A G T**	**2**
14316 Azerbaijan	v. *sabina*	*sabina*	A C G A T A G T	0
14317 Azerbaijan	v. *sabina*	*sabina*	A C G A T A G T	0
7811 Kazakhstan, Paniflor	**v. *sabina***	***sabina***	**R S R M Y M R K**	**8**
7812 Kazakhstan, Paniflor	v. *sabina*	*sabina*	G G A C C C A G	0
7585 Mongolia, Altair Mtns.	**v. *sabina***	***sabina***	**R S R M Y M R K**	**8**
7586 Mongolia, Altair Mtns	**v. *sabina***	***sabina***	**A C G A Y M R K**	**4**
7587 Mongolia, Altair Mtns	v. *sabina*	*sabina*	G G A C C C A G	0
7836 China, Heaven Lake, Xinjiang	**v. *sabina***	***sabina***	**R S R M Y M R K**	**8**
7837 China, Heaven Lake, Xinjiang	**v. *sabina***	***sabina***	**R S R M Y M R K**	**8**
most common sabina pattern	Type 1		G **G** A **C** C **C** A **G**	Type 1
7083, thurifera, France[2]			A **G** G **C** T C G G	0
9420, thurifera v. africana, Morocco[2]			A G **G C** T C G G	0
2nd most common sabina pattern	Type 2		**A** C **G** A **T** A **G** T	Type 2

[1]Eight polymorphic sites (1-8): R352, S391, R432, M606, Y745, M999, R1046, K1047.
350 xTGTCGGAG; 391 xGAGGTCCG; 432 xTCGTGTGC; 606 CGACAAGAx;
745(105) xCCAAAAGA; 999(333) xGCGAGGAG; 1046(392) xNGCGGTCGG;1047 xGCGGTCGG
[2]This pattern, **A G G C** *T* **C G G,** was also found in **ALL** *J. thurifera* samples examined, to date (14 *J. thurifera* samples from Corse, Morocco, France and Spain, Adams, et al. 2018)

Table 2. *Juniperus sabina* classified based on ITS homozygous for all 8 polymorphic sites. Putative hybrids (heterozygous for the 8 polymorphic sites) were excluded.

coll. #, location	trnS-trnG classification	ITS classif.	nrDNA type	polymorphic sites[1] 1 2 3 4 5 6 7 8	# sites in common with *J. thurifera*
14934 Spil Dagi, Turkey, Mataraci	v. *balkanensis*	*sabina*	1	G G A C C C A G	4
14728 Tsena Mtn., Greece	v. *balkanensis*	*sabina*	1	G G A C C C A G	4
15313 Mavrovo, Macedonia	v. *balkanensis*	*sabina*	1	G G A C C C A G	4
15319 Gaichnik, Macedonia	v. *balkanensis*	*sabina*	1	G G A C C C A G	4
15279 Mt. Cabulja, Bosnia-Herze.	v. *balkanensis*	*sabina*	1	G G A C C C A G	4
15280 Mt. Cabulja, Bosnia-Herze.	v. *balkanensis*	*sabina*	1	G G A C C C A G	4
15281 Mt. Cabulja, Bosnia-Herze.	v. *balkanensis*	*sabina*	1	G G A C C C A G	4
15343 Velebit, Croatia	v. *balkanensis*	*sabina*	1	G G A C C C A G	4
15344 Velebit, Croatia	v. *balkanensis*	*sabina*	1	G G A C C C A G	4
15508 Ceren, Albania	**v. *balkanensis***	***sabina***	**1**	**G G A C C C A G**	**4**
15508 Ceren, Albania	**v. *balkanensis***	***sabina***	**1**	**G G A C C C A G**	**4**
7197 Sierra Nevada, Granada, Spain	v. *sabina*	*sabina*	1	G G A C C C A G	4
7199 Sierra Nevada, Granada, Spain	v. *sabina*	*sabina*	1	G G A C C C A G	4
7573 Sallent deGallego, Spain	v. *sabina*	*sabina*	1	G G A C C C A G	4
7574 Sallent deGallego, Spain	v. *sabina*	*sabina*	1	G G A C C C A G	4
7812 Kazakhstan, Paniflor	v. *sabina*	*sabina*	1	G G A C C C A G	4
7587 Mongolia, Altair Mtns	v. *sabina*	*sabina*	1	G G A C C C A G	4,
7612 Switzerland	**v. *sabina***	***sabina***	**1**	**G G A C C C A G**	**4 Note Type 1!**
7614 Switzerland	**v. *sabina***	***sabina***	**2**	**A C G A T A G T**	**3, Note Type 2!**
14723 Rila Mtn., Bulgaria	v. *balkanensis*	*sabina*	2	A C G A T A G T	3
14316 Azerbaijan	v. *sabina*	*sabina*	2	A C G A T A G T	3
14317 Azerbaijan	v. *sabina*	*sabina*	2	A C G A T A G T	3
				bases in common (**bold**) w *thruifera*	
most common sabina pattern(type 1)			1	G **G** A **C** C **C** A **G**	4 sites in common
7083, thurifera, France, Morocco[2]				**A G G C T C G G**	8
2nd common sabina pattern(type 2)			2	**A** C **G** A **T** A **G** T	4 sites in common
14861 Spil Dağı, Turk., Boratynski	v. *balkanensis*	*sabina*		R G R C Y C R G	4
13725 eastern Rhodopes, Bulgaria	v. *balkanensis*	*sabina*		G G A M Y M R K	5
14722 Rila Mtn., Bulgaria	v. *balkanensis*	*sabina*		G G A C Y C A G	1
14724 Rila Mtn., Bulgaria	v. *balkanensis*	*sabina*		R G A C Y M A G	3
15320 Gaichnik, Macedonia	v. *balkanensis*	*sabina*		R C R A T M R K	5
15277 Mt. Cabulja, Bosnia-Herze.	v. *balkanensis*	*sabina*		R S R A Y M R K	6
15283 Mt. Biokovo, Croatia	v. *balkanensis*	*sabina*		G G A C Y M R K	4
14938 northeast Turkey Kandemir	v. *sabina*	*sabina*		R G R C T A G T	2
7586 Mongolia, Altair Mtns	v. *sabina*	*sabina*		A C G A Y M R K	4
most common sabina pattern)			Type 1	G G A C C C A G	
2nd common sabina pattern			Type 2	A C G A T A G T	
cross between *J. sabina* type1 x type2			1x2	R S R M Y M R K	
Putative 'hybrid' pattern (table 1)				R S R M Y M R K	

[1]Eight polymorphic sites (1-8): R352, S391, R432, M606, Y745, M999, R1046, K1047.
[2]This pattern, AGGCTCGG**,** was found in all *J. thurifera* samples examined, to date (14 *J. thurifera* samples from Corse, Morocco, France and Spain, Adams, et al. 2018)